

Supporting Information

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SI Methods

Protein Expression and Purification. A region of human BRCA2 corresponding to a 1,127-aa fragment between residues 987 and 2113 of the encoded protein was cloned into a pET28a vector (Novagen) and expressed in Rosetta (DE3) competent cells (Novagen) as described (1). Briefly, cells were lysed in 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM DTT containing protease inhibitors (Roche); the insoluble pellet was clarified and washed with 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1% Triton X-100, 5 mM DTT containing protease inhibitors (Roche). The insoluble material was solubilized in 50 mM Tris-HCl, pH 8.0, 8 M deionized urea, 5 mM DTT and purified under denaturing conditions by Q Sepharose HP resin (Amersham), the peak fractions dialyzed in 50 mM sodium acetate, pH 5.0, 8 M urea, 5 mM DTT, and finally the dialysate fractionated over SP Sepharose HP resin (Amersham) by NaCl gradient. Peak fractions were pooled and renatured by dialysis in 50 mM Tris-HCl, pH 8.0, 500 mM L-arginine, 10 mM DTT and subsequently transferred into 20 mM Tris-HCl, 10% glycerol, 5 mM DTT by thorough dialysis. The sample was concentrated using an Amicon Ultra-4 centrifugal filter device and stored at -80°C .

Full-length human RAD51 was cloned into a pET11d vector (Novagen), expressed in *recA⁻* cells BLR(DE3) competent cells (Novagen), and purified by a method previously described (2). Recombinant human RPA protein was made using the expressing vector p11d-tRPA (gift from Mark Wold, University of Iowa College of Medicine, Iowa City, IA) and purified as described (3). The protein was determined to be free from nuclease contamination. Protein concentrations were estimated by UV absorbance at 280 nm (Cary 50 spectrophotometer; Varian) with extinction coefficients estimated by ProtParam (4).

EMSA, Strand Exchange, and Restriction-Site Protection Assays. For EMSA, 3.3 μM RAD51 either in the absence or presence of increasing BRCA2_[BRCA1-8] concentration were assembled in Buffer A (40 mM Tris-HCl, pH 7.8, 1 mM MgCl_2 , 1 mM DTT, and 8 mM phosphocreatine) containing nucleotide cofactor (1 mM either ATP or AMP-PNP; see text) and KCl (200 mM final concentration) in 10- μL reaction volumes. After incubation at 37°C for 15 min, γ - ^{32}P -radiolabeled DNA substrates such as 10 μM resected dsDNA preassembled with RPA (0.07 μM ; Fig. S1) or 7 μM γ - ^{32}P -radiolabeled *Apa*LI-linearized double-stranded (lds) ϕX174 were added to the mixture and incubated for a further 60 min.

The strand exchange assay was performed as described for the EMSA, except that 3.3 μM RAD51 was preincubated with 1.2 μM BRCA2_[BRCA1-8] in Buffer A supplemented with KCl, ATP, and creatine phosphokinase (25 $\mu\text{g}/\text{mL}$; ATP regeneration) and γ - ^{32}P -radiolabeled resected dsDNA (10 μM)/RPA (0.07 μM) mixture for 15 min at 37°C . Unlabeled 7 μM *Apa*LI linearized-double-stranded (lds) ϕX174 was added, and samples removed for further analysis at specific time periods are indicated in the text and figure legends. Samples were deproteinized by addition of SDS/EDTA (0.2%/5 mM final concentration) and 600 $\mu\text{g}/\text{mL}$ proteinase K and incubation at 37°C for a further 15 min.

The protein-DNA complexes by EMSA were resolved by 1% (resected dsDNA) or 0.5% (*Apa*LI dsDNA) agarose gel electrophoresis in TAE buffer at 4°C . Deproteinized strand-exchange products were separated by 1.5% agarose gel electrophoresis in TAE buffer. All radioactive gels were dried and exposed to Phosphorimager screens, with data processed on a Fuji FLA-5000 scanner.

Dual EMSA/Restriction Site Protection Assay. EMSA reactions were assembled using γ - ^{32}P *Apa*LI-digested ϕX174 dsDNA. At the indicated times, 5 μL were mixed with Orange-G DNA loading buffer and kept on ice. The other half was treated with 10 U *Afl*III restriction enzyme at 37°C for a further 15 min and deproteinized as described above. The EMSA products were resolved by 0.5% agarose gel electrophoresis, whereas the restriction digest fragments were resolved on 1.2% agarose gel.

Preparation of Negative Staining EM Grids. Negatively stained EM grids were prepared according to standard procedures. Mesh copper EM grids (400) were used. A thin layer of carbon film (≈ 10 nm thick) was floated on distilled water and picked up on EM grids. Commercial carbon film-coated EM grids (Agar Scientific Ltd) were also used. The grids were then glow-discharged for 25 s in air before use to improve surface hydrophilicity. Two microliters protein-DNA solution (diluted 1:10) were applied to an EM grid for 3 min, blotted, then washed with a drop of Buffer A followed by staining with 2 successive drops of 2% uranyl acetate aqueous solution for 20 s and a final blotting with filtered-paper from the side of the grid. The grids were examined under low-dose conditions at room temperature on an FEI Tecnai 12 transmission electron microscope. The accelerating voltage was 120 kV, and the nominal magnification was between 26,000 and 42,000. Images were recorded on Kodak SO-163 film and developed in a Kodak D-19 developer at full strength for 12 min at 21°C . The micrographs were digitized by using the KZA densitometer (5).

Enumeration of Oligomeric RAD51 Rings by EM. For an estimation of the degree of reduction in oligomeric RAD51 rings found after the addition of BRCA2_[BRCA1-8], the average numbers of oligomeric RAD51 rings per unit area were compared. From 6 micrograph images each of RAD51-ssDNA and of BRCA2_[BRCA1-8]-RAD51-ssDNA, oligomeric RAD51 rings were counted using Ximdisp (6). A total of 1,029 oligomeric RAD51 rings were counted for RAD51-ssDNA, which corresponded to a total area of 98 μm^2 (10.54 oligomeric RAD51 rings/ μm^2) compared to 34 oligomeric rings for BRCA2_[BRCA1-8]-RAD51-ssDNA covering 38 μm^2 (0.96 oligomeric rings/ μm^2). Thus, an 11-fold reduction in oligomeric RAD51 rings was observed in the presence of BRCA2_[BRCA1-8].

Two-Color Coincidence Detection. HPLC-purified 5'-Alexa488-ssDNA was purchased (IBA Biotech GmbH) and consisted of 60-nucleotide ssDNA (TAC TGC CAT TCT GTA TCG CTT ATC GAG TAG TTA CCT GCC TAG CAT TGC CAC TCA TAG CCT). Alexa488-dsDNA was prepared by hybridizing Alexa488-ssDNA with an equal concentration of unlabeled complementary 60-nucleotide oligomer. Successful hybridization was confirmed by fluorescence correlation spectroscopy (FCS).

RAD51-Atto647N was prepared by labeling with Atto647N NHS Ester (Sigma). Labeling reactions were carried out in $1\times$ PBS buffer, pH 8.0, for 30 min, and then excess dye was removed using a Bio-Spin desalting column (Bio-Rad Laboratories). A 10-fold molar excess of RAD51 was used to ensure that no free dye was present in the product. Because the labeling was substoichiometric, absolute stoichiometries could not be inferred in the single-molecule experiments.

Briefly, overlapping beams from a blue laser (488-nm Solid State laser, model Cyan CW; Newport) and a red laser (633-nm

HeNe laser, model 25LHP151; Melles Griot) were directed into a high numerical aperture oil-immersion objective (Fluor 100×, NA 1.30; Nikon). Fluorescence signals were collected through the same objective and separated by a dichroic mirror (585DRLP; Omega Filters) before detection by a pair of avalanche photodiodes (SPCM AQR-141; EG&G). Laser powers of 300 μ W for the blue laser and 100 μ W for the red laser were used to give comparable fluorescence intensities from both fluorophores. Data were collected in 1-ms time bins for 6 min at 20 °C immediately after the dilution of the sample. Data were filtered to exclude frames containing large fluorescent aggregates of RAD51 with a burst duration exceeding 25 ms. These aggregates were infrequently sampled and showed no coincidence with DNA. The fraction of Alexa488-ss/dsDNA bound to RAD51-Atto647N was calculated as described (7) using the equation:

$$\text{Fraction of DNA bound} = \frac{r_C - r_E}{r_B}$$

where r_C is the rate of occurrence of coincident events, r_E is the expected rate of chance coincident events and r_B is the overall rate of blue fluorescent bursts observed from Alexa488 on DNA. The rate of chance coincident events is calculated as shown previously (8). To correct for incomplete overlap between the focal volumes of the 2 lasers, the fraction of DNA bound was calculated from a reference sample that was 100% hybridized to a complementary oligonucleotide, 5'-end-labeled with Atto647N.

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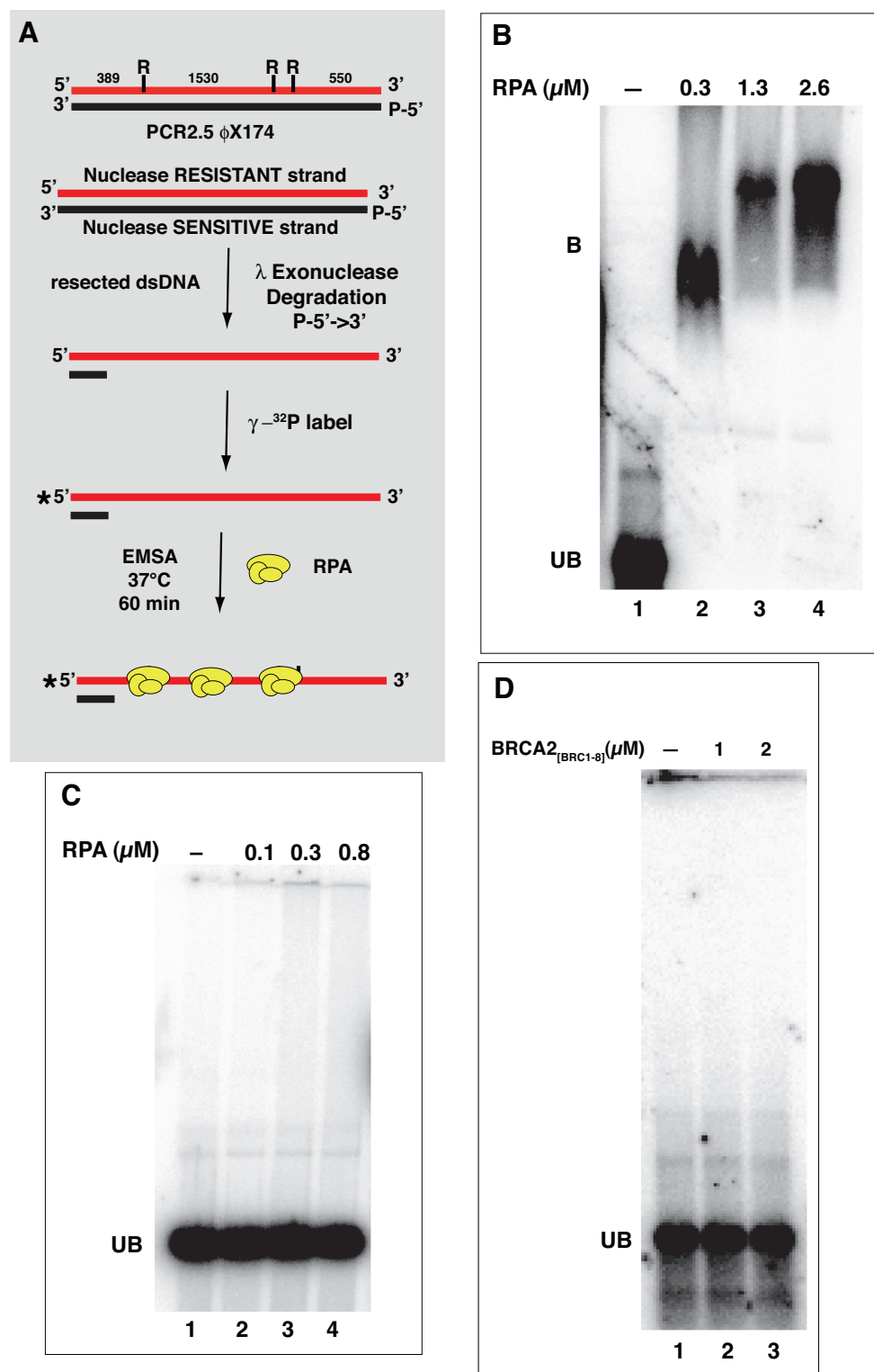


Fig. S1. Preparation and validation of the resected dsDNA substrate. (A) Strategy for generating resected dsDNA. Bacteriophage Φ X174 RF1 DNA (nucleotides 2799–5386), with 3 diagnostic *RsaI* restriction endonuclease sites, was amplified by PCR using a nuclease-resistant forward primer incorporating 5'-phosphorothioate chemistry, with a standard 5'-phosphorylated reverse primer. λ Exonuclease digestion of the nonresistant strand (black) creates a resected dsDNA. The resected dsDNA was radiolabeled with ^{32}P at the 5'-end and used as an EMSA probe to test its ability to interact with the ssDNA binding protein, RPA. (B) Increasing concentrations of RPA were incubated with 10 μM radiolabeled resected dsDNA for 60 min at 37 $^{\circ}\text{C}$ before EMSA analysis. (C) A similar EMSA assay using 7 μM radiolabeled dsDNA serves as a control. (D) Increasing concentrations of BRCA2_[BRCA1–8] (1.0 and 2.0 μM ; lanes 2 and 3) do not alter the EMSA mobility of the 10 μM radiolabeled resected dsDNA, indicating that BRCA2_[BRCA1–8] does not bind directly to this DNA substrate.

